

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Hein et al.

Title: METHOD OF USE OF
TRANSGENIC PLANT EXPRESSED
ANTIBODIES
(as amended)

Appl. No.: 09/491,332

Filing Date: 01/25/00

Examiner: C. Collins

Art Unit: 1638

DECLARATION OF ANDREW HIATT, Ph.D, UNDER 37 C.F.R. § 1.132

I, Andrew Hiatt, hereby declare that:

1. I was educated at the University of Vermont where I received a B.A. degree and at Colombia University where I received a Ph.D. degree. I was a postdoctoral fellow in the Delbruck Laboratory at the Cold Spring Harbor Research Laboratory and an assistant professor at the Scripps Clinic and Research Foundation. I have conducted research in plant biotechnology for over 20 years and am the author or co-author of over 30 published scientific articles in biomedical science. A brief summary of my accomplishments and a recent copy of my Curriculum Vitae is attached as APPENDIX 1. I am a founder and the chief scientific officer of Epicyte Pharmaceutical, Inc., a company focused on technology for expressing antibody products in plants. I am named as a co-inventor of the above-identified patent application. I should also mention that Epicyte Pharmaceutical, Inc. is an exclusive licensee of this patent application.

2. I understand that the Examiner has rejected the claims as being allegedly obvious over a doctoral dissertation by Klaus During ("the During dissertation") and Goodman (U.S. Patent 4,956,282), in view of Stolle et al. (U.S. Patent 4,748,018). I also understand that the Examiner has reviewed a declaration by Dr. Richard Lerner, dated March 11, 2002, but has discounted its applicability because the During dissertation

allegedly is limited to problems associated with processing and assembly of full length antibodies and the claims are not commensurate in scope (which I understand to mean that the claims cover antibody fragments). I understand the Examiner's position to be that production of an antigen specific dual chain antibody fragment does not involve processing and assembly of the chains. I also understand that the Examiner is applying this same reasoning to discount the findings in the Lerner declaration on the prejudice in the art against the conclusions of During. I bring this declaration for the purposes of addressing what appear to be scientific misconceptions held by the examiner.

I. An antigen specific immunoglobulin fragments require processing and assembly.

3. The Examiner's assertion that the claims are not commensurate in scope because antigen specific immunoglobulin fragments of a fully assembled antibody (i.e., immunologically active antibody fragments) do not require processing and assembly is scientifically incorrect. It has long been known that an antigen specific immunoglobulin fragments, like the full length version of these antibodies, require processing and assembly. The following references are illustrative of this point.

Skerra et al., *Science* 240:1038-1041 (attached as APPENDIX 2) described assembly of a functional immunoglobulin Fv fragment in *E. coli* bacteria. In this regard, the abstract of Skerra on page 1038 states as follows (emphasis added):

An expression system was developed that allows the production of a completely functional antigen-binding fragment of an antibody in *Escherichia coli*. The variable domains of the phosphorylcholine-binding antibody McPC603 were secreted together into the periplasmic space, where **protein folding and as well as heterodimer association occurred correctly. Thus, the assembly pathway for Fv fragment of McPC603 is similar to that of a whole antibody in the eukaryotic cell.**

With respect to processing, Skerra et al. evaluated the N-terminal sequence of the heavy and light chain variable regions and concluded that the leader sequences were properly cleaved by bacterial signal peptidase. *Id.* at page 1040, left hand column. Skerra et al.

also showed that the expressed fragment was antigen specific. *Id.* Thus, it is evident from Skerra et al. an antigen-specific antibody fragment (like that of the full-length antibody) requires processing and assembly.

Better, *Science* 240:1041-1043 (attached as APPENDIX 3)

described assembly of a functional immunoglobulin Fab fragment in *E. coli* bacteria. The Fab fragment of Better et al. is a chimeric antibody which contains the variable regions of a mouse monoclonal antibody with the C_H1 and C_κ constant regions from a human IgG1 immunoglobulin. Better et al. concluded from an evaluation of antibody Fab fragments produced in culture supernatants that a processed and fully assembled fragment was produced. *Id.* at page 1042, middle column ("These observations of are consistent with the predicted molecular sizes of the processed chimeric Fab . . . and suggest that the material is properly processed."). Better et al. also showed that the expressed Fab fragment was antigen specific. *Id.* Thus, it is evident from Better et al. that an antigen-specific antibody fragment (like that of the full-length antibody) requires processing and assembly.

Horowitz et al., *Proc. Natl. Acad. Sci. (USA)* 85:8678-8682

(attached as APPENDIX 4) described assembly of a functional full-length ("whole") chimeric antibody and a Fab fragment derived therefrom by yeast cells. The Fab fragment of Horowitz et al. is a chimeric antibody which contains the variable regions of a mouse monoclonal antibody with the C_H1 and C_κ constant regions from a human IgG1 immunoglobulin. Horowitz et al. concluded that processing and assembly of antigen specific full-length antibody and related Fab fragments occurred in yeast. *Id.* at page 8681, left hand column ("Several lines of evidence support the thesis that these proteins are correctly folded.").

4. Similar findings to those above had been previously reported for antibody expression in lymphoid cells (see Neuberger et al. *Nature* 312:604-608, 1984; attached as APPENDIX 5). Thus, the Examiner is mistaken in believing that the fragments of a fully assembled antibody do not require processing and assembly. Antigen specific dual chain antibodies, whether full-length or fragments thereof, have been shown

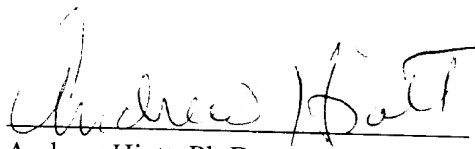
in mammalian and microbial systems to require correct processing and assembly for their production.

II. The prejudice in the art described in the Lerner declaration also applies to antigen-specific immunoglobulin fragments.

5. The Examiner belief that the findings of the Lerner declaration that a prejudice in the art existed circa 1988/1989 against producing a processed and fully assembled antibody in plant cells would not have applied to the production of an antigen-specific antibody fragment in plant cells also is mistaken. It is evident from the above-discussed art that antigen-specific antibody fragments require processing and assembly as do a full-length antibody. Thus, it necessarily follows that the prejudice in the art against the producing full-length antibody in plant cells also would have applied equally well to an antigen-specific antibody fragment. In my view, the Lerner declaration states as such by referring to a "processed and assembled immunoglobulin" (Lerner declaration, ¶8), which is a term that encompasses an antigen-specific full length antibody and antigen specific antibody fragments.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the captioned patent application or any patent issued therefrom.

Feb. 19, 2003
Date


Andrew Hiatt, Ph.D.
EpicYTE Pharmaceutical, Inc.
5810 Nancy Ridge Dr.
Suite 150
San Diego, CA 92121

APPENDIX 1

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME HIATT, Andrew		POSITION TITLE V.P., Research & Development	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Vermont, Burlington, VT	B.A.	1973	Biology
Columbia University, New York, NY	Ph.D.	1983	Endocrinology / Biochemistry

Professional Experience:

1996 - present Vice President, R&D, Epicyte Pharmaceutical, Inc., San Diego, California
 1994 - 1996 Research Director, Rose-Hiatt Biotechnology L.L.C., San Diego, California
 1987 - 1994 Assistant Professor, The Scripps Research Institute, La Jolla, California
 1986 - 1987 Staff Investigator, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
 1984 - 1986 Damon Runyon/Walter Winchell Cancer Fund Fellowship, Cold Spring Harbor Lab.
 1978 - 1983 NIH Endocrinology Traineeship, Columbia University, New York, NY
 1980 - 1982 Biochemistry Teaching Assistantship, Columbia University, New York, NY

Publications (Selected)

- Vine, N.C., Drake, P., Hiatt, A., and Ma, J. K-C. Assembly and Plasma Membrane Targeting of Recombinant Immunoglobulin Chains in Plants with a Murine Transmembrane Sequence. 2001. Plant Molecular Biology 45:159-167.
- Ma, J. K-C., Hiatt, A., M. Hein, N. Vine, K. Mostov, C. van Dolleweerd, P. Stabila and T. Lehner. 1995. Assembly of Secretory Antibodies in Transgenic Plants. Science, 268: 716-719.
- Ma, J. K-C., Lehner, T., Stabila, P., Fux, C.I., and Hiatt, A. 1994. Assembly of Monoclonal Antibodies with IgG1 and IgA Heavy Chain Domains in Transgenic Tobacco Plants. Eur. J. Immunol., 24: 131-138.
- Hiatt, A., Merlock, R., Mauch, S., Wrasidlo, W. 1994. Regulation of apoptosis in leukemic cells by analogs of dynemicin A. Bioorg Med Chem 2(5):315-322.
- Nicolaou, K.C., Stabila, P., Esmaeli-Azad, B., Wrasidlo, W., and Hiatt, A. 1993. Cell Specific Regulation of Apoptosis by Designed Enediynes. Proc. Natl. Acad. Sci. USA 90: 3142-3146.
- Hiatt, A., and Hein, M., 1993. Structure, Function, and Uses of Antibodies from Transgenic Plant and Animals, in "Handbook of Experimental Pharmacology" M. Rosenberg and M. Gordon eds., Springer Verlag.
- Hiatt, A., and Ma, J. K.-C. 1992. Characterisation and Applications of Antibodies Produced in Plants, in "Antibody Engineering: Medical Applications", M. Zanetti ed., Gordon and Breech Publishers.
- Hiatt, A., Tang, Y., Weiser, W., and Hein, M.B. 1992. Assembly of Antibodies and Mutagenized Variants in Transgenic Plants and Plant Cell Cultures, in "Genetic Engineering, Principle and Methods, Vol. 14", J.K. Setlow ed., Plenum Press, 49-64.
- Hiatt, A. 1992. Assembly of Multimeric Proteins in Plant Cells: Characteristics and Uses of Plant-Derived Antibodies, in "Transgenic Plants: Fundamentals and Applications" A. Hiatt ed., Marcel Dekker, in press.
- Hiatt, A., and Ma, J. K.-C. 1992. Monoclonal Antibody Engineering in Plants. FEBS Letters, 307: 71-75.
- Hiatt, A. 1991. Monoclonal Antibodies, Hybridoma Technology and Heterologous Production Systems. Current Opinion in Immunology, 3: 229-232.
- Hiatt, A., and Pinney, R.P. 1991. Plantibodies: Expression of Monoclonal Antibodies in Plants, in "Antibody Engineering", C.A.K. Borrebaeck ed., Stockton Press, pp. 159-176

13. Hein, M., Tang, Y., McLeod, D.A., Janda, K.D. and Hiatt, A.C. 1991. Evaluation of Immunoglobulins from Plant Cells. *Biotechnology Progress*, 7:455-461.
14. Hiatt, A. 1991. Production of Monoclonal Antibody in Plants. *Transplantation Proceedings* 23: 147-151.
15. Hiatt, A. Antibodies Produced in Plants. 1990. *Nature*, 344: 469-470.
16. Hiatt, A., Cafferkey, R. and Bowdish, K. 1989. Production of Antibodies in Transgenic Plants. *Nature* 342: 76-78.
17. Hiatt, A.C. 1989. Polyamine Synthesis in Maize Cell lines. *Plant Physiol.*, 90: 1378-1381.
18. Hiatt, A.C. and Malmberg, R.L. 1988. Utilization of Putrescine in Tobacco Cell Lines Resistant to Inhibitors of Polyamine Synthesis. *Plant Physiol.*, 86: 441-446.
19. Hiatt, A.C., McIndoo, J., Malmberg, R.L. 1986. Regulation of Polyamine Biosynthesis in Tobacco: Effects of Inhibitors and Exogenous Polyamines on Arginine, Ornithine, and S-Adenosylmethionine Decarboxylases. *J. Biol. Chem.*, 261: 1293-1298.
20. Malmberg, R.L. and Hiatt, A.C. 1986. Polyamines in Plant Mutants. In "The Physiology of Polyamines" (U. Bachrach, Y. Heimer eds.). CRC Press Inc., Boca Raton, FL, pp.148-159.
21. Malmberg, R.L., McIndoo, J., Hiatt, A.C., Lowe, B. 1985. Polyamine Mutants and Developmental Switches in the Tobacco Flower. *Cold Spring Harbor Symp. Quant. Biol.*, Vol. L: 475-482.
22. Hiatt, A.C., McDonough, A.A., Edelman, I.S. 1984. Assembly of the (Na⁺ +K⁺)-Adenosine Triphosphatase: Post-Translational Membrane Integration of the α -Subunit. *J. Biol. Chem.* 259: 2629-2635.
23. McDonough, A.A., Hiatt, A.C., Edelman, I.S. 1983. Antibodies to Na/K-ATPase: Characterization and Use in Cell-Free Synthesis Studies. *Current Topics in Membranes and Transport* 19: 787-789.
24. Edelman, I.S. and Hiatt, A.C. 1983. Structure and Biogenesis of the Na/K Pump. In "Biological Structures and Coupled Flow" E. Katzir-Katchalsky ed., Rehovot Israel, PP. 421-428.
25. McDonough, A.A., Hiatt, A.C., Edelman, I.S. 1982. Characterization of Antibodies to Guinea Pig Na/K-ATPase and Their Use in Cell-Free Synthesis Studies. *J. Membrane Biology* 69: 13-22.

APPENDIX 2

sera from humans in endemic areas (30) and may play a role in protective immunity (19). This suggests that p41 is exposed to the host's immune system at a certain point of the infectious cycle, probably when the content of the rhoptry organelles is secreted onto the erythrocyte membrane (31). At this step, antibodies could inactivate the rhoptry proteins that are believed to participate in the invasion process (7, 19).

Certain features of the p41 sequence suggest two independent approaches to the control of malaria. First, the need for functional aldolase may reflect the strong conservation of the sequence among different isolates; it is unlikely that parasite mutants evolve that escape immune attack by protective antibodies against p41. Second, the common function of the enzyme yet the relatively high degree of sequence diversity between human and parasite aldolase suggests the possibility of finding compounds that specifically inhibit the parasite enzyme.

21. U. Gubler and B. J. Hoffman, *Gene* 25, 263 (1983).
22. T. V. Huynh, R. A. Young, R. W. Davis, in *DNA Cloning: A Practical Approach*, D. M. Glover, Ed. (IRL Press, Washington, DC, 1985), vol. 1, pp. 49-78.
23. U. Certa, D. Rotmann, H. Matile, R. Reber-Liske, *EMBO J.* 6, 4187 (1987).
24. The isolates came from East Africa (RO-58, RO-59, FCH 5-C2, and Palo Alto), West Africa (R-PCR 3, RO-33, Geneva no. 13, and RO-53), Central America (H-B3), South America (542), Asia (K1, CPG-1, T9/94, and NT-112), and the South Pacific (MAD-20).
25. J. Devereux, P. Haeblerli, O. Smithies, *Nucleic Acids Res.* 12, 387 (1984).
26. R. K. Wicanga et al., *EMBO J.* 6, 215 (1987).
27. C. Y. Lai and B. L. Horecker, *Essays Biochem.* 8, 149 (1972).
28. R. W. McKee, in *Biochemistry and Physiology of Protozoa*, S. H. Hutner and A. Lwoff, Eds. (Academic Press, New York, 1951), vol. 1, pp. 251-322.
29. N. Saxena, V. C. Pandey, G. P. Dutta, S. Ghatak, *Indian J. Exp. Biol.* 24, 502 (1986).
30. M. A. Gabra et al., *Bull. WHO* 64, 389 (1986).
31. L. H. Bannister, G. H. Mitchell, G. A. Butcher, E. D. Donnis, *Parasitology* 92, 291 (1986).
32. L. H. Perrin, unpublished data.
33. K1 parasites were cultivated and extensively washed as described (23). The parasites were lysed by eight cycles of freeze-thawing. Lysis was monitored microscopically. Membranes and debris were removed by centrifugation at 12,000 rpm for 10 min at 4°C.

Aliquots of this lysate or purified p41 protein (3) containing 50 units (1 unit is 1 μ mol of dihydroxyacetone phosphate formed per minute and per milligram of protein at 37°C) of aldolase were assayed with a commercial aldolase kit (Sigma, procedure 752). For inhibition, 25 μ l of rabbit antiserum to p41 (11) or preimmune serum were preincubated with the sample for 5 min at 25°C.

34. The increment in the preimmune serum control is due to aldolase and triose-phosphate isomerase activity present in rabbit serum.
35. Sizing of active p41 was performed on a 60-cm LKB TSK-G 3000 SW HPLC column connected to a 7.5-cm precolumn in 0.1M sodium phosphate, pH 7.5, at a flow rate of 0.4 ml/min. The column was calibrated with leucine aminopeptidase (326 kD), rabbit muscle aldolase (160 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), human immune interferon (32 kD), myoglobin (18 kD), and cytochrome c (12 kD). Proteins were detected after α -phthalaldehyde derivatization (36).
36. M. Roth, *Anal. Chem.* 43, 880 (1971).
37. U. K. Laemmli, *Nature* 227, 680 (1970).
38. We thank D. Doran for computer-assisted screening of the NBRF database; Y. Burki and D. Rotmann for technical assistance; S. LeGrice, K. Brenzel, and R. Genz for discussions; and E. Kawashima for synthetic DNA adapters. Parts of this work were supported by the Swiss National Research Foundation.

22 December 1987; accepted 15 March 1988

REFERENCES AND NOTES

1. W. A. Siddiqui, *Science* 197, 388 (1977).
2. G. H. Mitchell, G. A. Butcher, W. H. G. Richards, S. Cohen, *Lancet* i, 1335 (1977).
3. L. H. Perrin et al., *J. Clin. Invest.* 76, 1718 (1985).
4. L. H. Perrin and R. Dayal, *Immunol. Rev.* 61, 245 (1982).
5. R. F. Howard, H. A. Stanley, G. H. Campbell, R. H. Reese, *Am. J. Trop. Med. Hyg.* 33, 1055 (1984).
6. C. Braun-Brenton et al., *Mol. Biochem. Parasitol.* 20, 33 (1986).
7. L. Schofield, *ibid.* 18, 183 (1986).
8. R. R. Hall et al., *Nature* 311, 379 (1984).
9. R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* 80, 1194 (1983).
10. R. A. Young et al., *ibid.* 82, 2553 (1985).
11. Affinity-purified p41 protein (3) was injected into rabbits according to standard immunization protocols. When parasite lysates are probed in immunoblots, a single band of 41 kD is detected by the serum.
12. W. D. Benton and R. W. Davis, *Science* 196, 180 (1977).
13. The sequence data have been submitted to the European Molecular Biology Laboratory/GenBank data libraries under accession number J03084.
14. K. Tanabe, M. Mackay, M. Goman, J. G. Scalf, *J. Mol. Biol.* 196, 273 (1987).
15. D. J. Kemp et al., *Parasitology* 91, 83 (1986).
16. Peptides were generated by digestion of 80 μ g of affinity-purified p41 protein (3) with *Staphylococcus aureus* protease V8 and separated by reversed-phase high-performance liquid chromatography (HPLC) by using 0.1M trifluoroacetic acid and an acetonitrile gradient as the mobile phase. The amino acid sequences were determined on an Applied Biosystems 470 A gas phase sequencer.
17. C. Yanish-Perron, J. Vieira, J. Messing, *Gene* 33, 103 (1985).
18. The Eco RI insert of clone M25-13 was subcloned in the proper orientation into pUC18 (17), resulting in pGF-2. A 1-liter overnight culture of *E. coli* transformed with pGF-2 was concentrated by centrifugation, and the bacterial pellet was dissolved in 5 ml of lysis buffer (3) supplemented with lysozyme (10 mg/ml). When lysis was apparent, further purification steps were carried out as described (3), yielding 5 μ g of 41-kD recombinant protein. ELISA assays were performed as described (22).
19. L. H. Perrin, E. Ramirez, P. H. Lambert, P. A. Miescher, *Nature* 289, 301 (1981).
20. K. G. Odink, S. C. Nicolls, A. J. Lockyer, Y. Hillman, *Mol. Biochem. Parasitol.* 10, 55 (1984).

Assembly of a Functional Immunoglobulin F_v Fragment in *Escherichia coli*

ARNE SKERRA AND ANDREAS PLÜCKTHUN*

An expression system was developed that allows the production of a completely functional antigen-binding fragment of an antibody in *Escherichia coli*. The variable domains of the phosphorylcholine-binding antibody McPC603 were secreted together into the periplasmic space, where protein folding as well as heterodimer association occurred correctly. Thus, the assembly pathway for the F_v fragment in *E. coli* is similar to that of a whole antibody in the eukaryotic cell. The F_v fragment of McPC603 was purified to homogeneity with an antigen-affinity column in a single step. The correct processing of both signal sequences was confirmed by amino-terminal protein sequencing. The functionality of the recombinant F_v fragment was demonstrated by equilibrium dialysis. These experiments showed that the affinity constant of the F_v fragment is identical to that of the native antibody McPC603, that there is one binding site for phosphorylcholine in the F_v fragment, and that there is no inactive protein in the preparation. This expression system should facilitate future protein engineering experiments on antibodies.

IMMUNOGLOBULINS (IGs) ARE A FAMILY of stable and similar molecules that can bind to a large number of different antigens. They constitute promising targets for investigating protein-ligand interactions since the overall folding of the domains seems to be independent of the structure of the binding site. Several three-dimensional structures of antibodies or their Fab fragments have been determined, and their common features have been compared (1). The essence of their architecture is a framework of fairly constant residues (arranged in a sandwich of β -sheets) linked by three hyper-variable loops [complementarity-determin-

ing regions (CDR)] per chain that determine the specificity for antigen recognition. An early insight into enzyme catalysis by Pauling (2) was used to search for catalytic antibodies (3). An easy access to genetically engineered, functional antibody proteins would permit new approaches for studying antibody structure and function and the essentials of enzymatic catalysis (4).

Despite numerous investigations, the

Genzentrum der Universität München, Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, FRG.

*To whom correspondence should be addressed.

expression of functional whole antibodies or functional antigen-binding fragments of antibodies has not been reported for any bacterial expression system, and the chance of designing such an expression system has been viewed pessimistically (5, 6). The expression of antibodies in yeast has been described (7), but only a small fraction of the expressed protein was functional. The purification of active antibodies or antibody fragments from yeast or any other microorganism has not been reported. In *E. coli*, the antibody protein could be produced only in a non-native state (8), and refolding experiments led to only a small percentage of correctly folded recombinant antibodies. Moreover, it is difficult to purify the native protein from non-native contaminants, which complicates accurate measurements of binding constants, folding yields, and spectral properties.

Other investigators preferred expression in cells of higher eukaryotes, thus permitting

the production of functional antibodies (9, 9, 10). Yet none of these expression systems compares to *E. coli* in the ease of genetic manipulation, efficient transformation, fast growth, simple fermentation, and favorable economics. A bacterial expression system in which the chains assemble to form a functional complex in the same cell would permit the use of assays directly on bacterial colonies. One could then use positive selection methods for antigen binding and possible catalytic functions of the mutant antibodies. Apart from the investigation of the variable regions themselves, the possible replacement of the constant regions through genetic means by marker enzymes (11), toxins (12), or Ig regions from a different class (13) or from a different species (14) has attracted attention. The production of such hybrid antibodies may also be facilitated by a bacterial expression system.

In our search for an antibody model for investigations on binding and catalysis, we

decided on an antibody with a known amino acid sequence and a three-dimensional structure that may also be close to a transition-state binding protein. Such a system is the myeloma protein McPC603 (15, 16), a phosphorylcholine-binding IgA₁ from mouse. We decided to investigate the expression of the F_v fragment of McPC603 in *E. coli*. This fragment is the dimer of the V_L (115 amino acids) and V_H (122 amino acids) domains and contains the whole antigen binding site. Each domain has one intramolecular disulfide bond (connecting Cys-23 to Cys-94 in V_L and connecting Cys-22 to Cys-98 in V_H). There is no disulfide bond between the chains and no other free cysteine. We synthesized the genes for both the V_L and the V_H domain. The exact DNA sequence we synthesized, the synthesis methodology, and the logic of the sequence design are discussed elsewhere (4).

The expression system described herein is the result of attempts to reproduce in *E. coli* the folding and assembly pathway of antibodies in eukaryotic cells. In the eukaryotic plasma cell, the two chains of an antibody are separately transported from the cytoplasm to the lumen of the endoplasmic reticulum (ER) (17). This transport requires an NH₂-terminal signal sequence, which is cleaved off during or after the translocation event by a signal peptidase, to produce the mature protein. In the lumen of the ER, protein folding, formation of the disulfide bonds, and the association of individual chains to form the functional antibody take place (17). It is not clear yet which other proteins play an essential role in mediating these folding and assembly processes. In addition to these critical steps, which must be mimicked in the bacterial cell in order to obtain a functional F_v fragment, the antibodies are glycosylated in the lumen of the ER and Golgi apparatus and transported to the cell surface. Usually only the F₂ is glycosylated, but this glycosylation is not required for antigen binding.

Our hypothesis was that the protein transport to the periplasm of *E. coli* is functionally equivalent to the transport of a protein to the lumen of the ER of a eukaryotic cell. We developed a system for expressing both chains in the same *E. coli* cell and secreting them together into the periplasm of *E. coli*. This system should permit the following critical steps in the assembly of a functional F_v fragment to occur: (i) synthesis of approximately stoichiometric amounts of both chains, (ii) transport of both precursor proteins to the periplasmic space, (iii) correct processing of both signal sequences, resulting in the same NH₂-termini as in the protein isolated from the mouse, (iv) fold-

Fig. 1. Plasmid pASK22 for the coexpression and cosecretion of the V_L and V_H domain of McPC603 in *E. coli*. The plasmid contains the origin of replication (ori) and the ampicillin resistance gene (Ap^r) from the pUC family of plasmids (29). The V_H and the V_L domain are encoded as two separate proteins on the same transcription unit downstream from a *lac* promoter-operator (*lac*^{P/O}) (29), which is inducible by isopropyl-β-D-thiogalactoside (IPTG). The synthetic genes encoding the V_H and the V_L domain (4) are precisely fused to gene fragments encoding the signal sequence of the outer membrane protein A (*ompA*) (19) and the alkaline phosphatase (*phoA*) (20), respectively. Each coding region is preceded by a ribosomal binding site to ensure efficient translation initiation. Thus, both genes are arranged in a regulatable artificial dicistronic operon. The construction of the plasmid was performed by using standard DNA methodology (30).

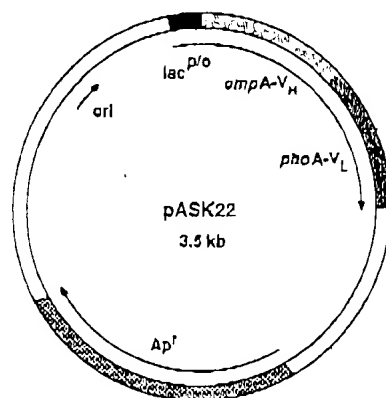
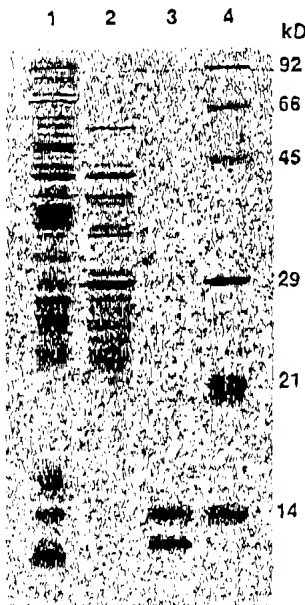


Fig. 2. Purification of the F_v fragment. An SDS-PAGE (14%) (31) stained with Coomassie brilliant blue is shown. (Lane 1) Total cell protein; (lane 2) periplasmic fraction; (lane 3) purified F_v fragment; and (lane 4) protein molecular size marker. For the purification, a culture of *E. coli* strain W3110 harboring plasmid pASK22 was grown in lactose broth (LB) medium containing ampicillin (100 mg/liter) to attain an absorbance at 550 nm of 0.5. After induction for 45 min by addition of isopropylthiogalactoside to a final concentration of 1 mM, the cells were harvested by centrifugation at 4000g for 10 min (at 4°C). A cell fractionation was carried out by resuspending the cell pellets in TES buffer (0.2M Tris-HCl, pH 8.0; 0.5 mM EDTA; 0.5M sucrose) (10 ml per liter of original culture). The cells were then subjected to a mild osmotic shock by addition of TES, diluted 1:4 with H₂O, and containing 2 mM phosphorylcholine (15 ml per liter of original culture). After incubation on ice for 30 min, the suspension was centrifuged (5000g, 10 min) and the supernatant was centrifuged again (48,000g, 15 min). This supernatant, which contained all soluble periplasmic proteins (32), was concentrated by ultrafiltration with an Amicon YM 5 membrane to a volume of approximately 2.5 ml per liter of original culture and dialyzed against BBS buffer (0.2M borate/NaOH pH 8.0, 0.16M NaCl). This concentrated solution was applied to a phosphorylcholine affinity column (2.5-ml bed volume per 4 liters of bacterial culture) (33). After washing with BBS, pure F_v fragment was eluted with a solution of 1 mM phosphorylcholine in BBS. The typical yield of this not yet optimized procedure is approximately 0.2 mg of purified F_v fragment per liter of bacterial culture.



ing to globular and soluble domains, (v) formation of the intramolecular disulfide bonds, and (vi) association of the two chains to form a heterodimer. Several examples (18) illustrate the secretion of heterologous monomeric proteins into the periplasm of *E. coli*, but it was not known whether folding and assembly of two different subunits can also occur to form a functional dimeric protein.

The expression vector we constructed is schematically drawn in Fig. 1. The genes, precisely fused to bacterial signal sequences (19, 20), are arranged in an artificial dicistronic operon. A homogeneous F_v fragment can be prepared from the periplasmic fraction of a cell lysate in a single step by affinity chromatography (legend to Fig. 2).

As can be deduced from the SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2), the F_v fragment is completely pure. Both chains of the purified F_v fragment are present in a 1:1 molar ratio and have the sizes expected for the mature proteins (V_H , 13,600; V_L , 12,400). To confirm the correct cleavage of both signal sequences, the six amino-terminal amino-acids of the two chains [V_H , NH_2 -Glu-Val-Lys-Leu-Val-Glu; V_L , NH_2 -Asp-Ile-Val-Met-Thr-Gln; (15)] were sequenced (21, 22). Both heterologous fusion proteins were properly cleaved by the bacterial signal peptidase, and there was no indication of either imprecise processing or any NH_2 -terminal degradation.

We measured the affinity constant of the recombinant F_v fragment for phosphorylcholine by equilibrium dialysis (Fig. 3). The same conditions were used as in the determination of the affinity constant of native McPC603 isolated from mouse ascites (23). The value found for the F_v fragment ($1.21 \pm 0.06 \times 10^5 M^{-1}$) (Fig. 3) is identical (within experimental error) to that reported (23) for the native antibody ($1.6 \pm 0.4 \times 10^5 M^{-1}$). The Scatchard plot (Fig. 3) is linear and extrapolates to approximately 1 mole of hapten bound per mole of F_v fragment. This shows that there is one binding site per F_v fragment and that there is no inactive protein in the preparation.

We conclude that it is possible to express the F_v fragment of McPC603 as a fully functional and stable protein in *E. coli*. There was no previous indication of whether *E. coli* would be able to assemble a protein consisting of different subunits in the periplasm. *Escherichia coli* seems to assemble its own proteins by a different method. *Escherichia coli* penicillin-acylase, the best characterized protein that fits the definition of a soluble heterodimeric protein in the periplasm, is proteolytically processed from a single chain precursor in the periplasm (24). Our results

indicate that folding and hetero-association of the variable domains is possible without known external help and is strongly favored in the periplasm of *E. coli*. Thus we could show that even when the assembly of two different chains is necessary for the formation of a functional protein, the transport to the periplasm of *E. coli* is functionally equivalent to the eukaryotic transport to the lumen of the ER. Further experimentation will be needed to clarify whether there is any role of homodimers (Bence-Jones proteins) (25) as assembly intermediates, or whether the correct heterodimer association is both kinetically and thermodynamically favored over homodimer formation. Most of the soluble V_L and V_H protein from the periplasmic fraction binds to the affinity column, indicating that it is correctly assembled to a heterodimer.

Our second result is that the F_v fragment of McPC603 has essentially the same affinity constant for phosphorylcholine as the intact

antibody McPC603. This finding could not be expected a priori, since there is considerable debate about the functionality of F_v fragments (26, 27). The first accurate study of an F_v fragment focused on the dinitrophenol (DNP)-binding antibody MOPC-315. It revealed that the affinity constants for DNP were essentially identical for the F_v fragment and the Fab fragment (26). In a recent investigation of the human riboflavin-binding antibody Gar (27), a fragment consisting of V_H and the whole light chain was prepared. This fragment, which is comparable to an F_v fragment, has an affinity constant for riboflavin that is about three orders of magnitude lower than that determined for the native antibody. These results were contradictory, and it was not clear whether they are the consequence of true differences between antibodies (28) or are the result of experimental side effects (27).

We conclude that the F_v fragment of McPC603 is fully functional and can serve as a convenient model for studying antigen-antibody interactions, since the three-dimensional structure of the corresponding Fab fragment is known (16). We have devised an expression system not requiring any in vitro manipulations such as cleavage of fusion proteins, oxidation, or refolding. Furthermore, expression in a functional state permits the use of hapten binding for rapid and selective purification. The periplasmic location of the protein reduces both the potential protease degradation problem and the number of contaminating protein species to be separated. We believe that protein engineering of antibodies is greatly facilitated with this expression system.

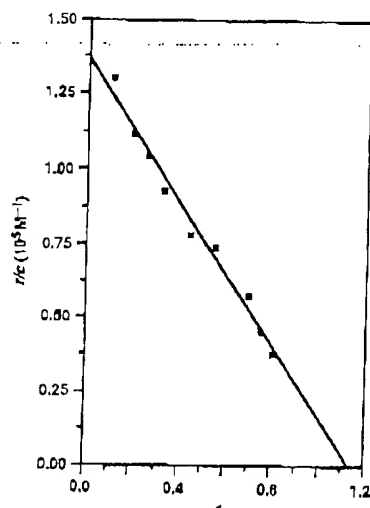


Fig. 3. Equilibrium dialysis data for the binding of phosphoryl[^{14}C]choline to the recombinant F_v fragment of McPC603. The equilibrium dialysis was carried out in a multicavity microdialysis chamber (Bel-Art Products) with a volume of about 100 μ l on each side of the membrane. The chambers were filled with 50 μ l of purified F_v fragment in BBS on one side and 50 μ l of a solution of phosphoryl[^{14}C]choline (50 mCi/mmol, Amersham) in BBS on the other side. The concentration of the F_v fragment was determined to be 0.22 mg/ml from an A_{205} of 6.85 [using an extinction coefficient of $\epsilon_{1cm}^{mg/ml} = 31$ for the average absorption of the peptide bond region in proteins at 205 nm (34)]. After equilibration for 22 hours at ambient temperature, samples (20 μ l) from each solution were counted in 5 ml of Rotiszint 22 (Roth Biochemicals) using a Beckman LS 1801 scintillation counter. The data obtained are plotted according to Scatchard (35). An r denotes the fraction of antibody fragment with bound hapten, and c denotes the concentration of free hapten. The line was fitted by linear regression analysis. From the slope of this line an affinity constant of $K_a = 1.21 (\pm 0.06) \times 10^5 M^{-1}$ is obtained.

REFERENCES AND NOTES

1. E. A. Kabat, *Adv. Protein Chem.* 32, 1 (1978); L. M. Amzel and R. J. Poljak, *Annu. Rev. Biochem.* 48, 961 (1979); D. R. Davies and H. Metzger, *Annu. Rev. Immunol.* 1, 87 (1983).
2. L. Pauling, *Chem. Eng. News* 24, 1375 (1946).
3. V. Raso and B. D. Stollar, *Biochemistry* 14, 584 (1975); *ibid.*, p. 591; A. Tramontano, K. D. Janda, R. A. Lerner, *Science* 234, 1566 (1986); S. J. Pollack, J. W. Jacobs, P. G. Schultz, *ibid.*, p. 1570; J. Jacobs, P. G. Schultz, R. Sugawara, M. Powell, *J. Am. Chem. Soc.* 109, 2174 (1987).
4. A. Plückhahn, R. Glockshuber, I. Pützinger, A. Skerá, J. Stedlmüller, *Cold Spring Harbor Symp. Quant. Biol.*, in press.
5. S. L. Morrison, *Science* 229, 1202 (1985).
6. M. A. Boss and C. R. Wood, *Immunol. Today* 6, 12 (1985).
7. C. R. Wood *et al.*, *Nature* 314, 446 (1985).
8. S. Cabilly *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 81, 3273 (1984); M. A. Boss, J. H. Kenren, C. R. Wood, J. S. Entage, *Nucleic Acids Res.* 12, 3791 (1984).
9. S. L. Morrison and V. T. Oi, *Annu. Rev. Immunol.* 2, 239 (1984).
10. S. Roberts and A. R. Rees, *Protein Eng.* 1, 59 (1986).
11. M. S. Neuberger, G. T. Williams, R. O. Fox, *Nature* 312, 604 (1984).
12. G. Möller, Ed., *Immunol. Rev.* 62 (1982).
13. M. S. Neuberger *et al.*, *Nature* 314, 268 (1985).

14. P. T. Jones, P. H. Dear, J. Foote, M. S. Subberger, G. Winter, *ibid.* 321, 522 (1986).
15. M. Potter, *Adv. Immunol.* 25, 141 (1977); R. M. Perlmutter *et al.*, *ibid.* 35, 1 (1984); S. Rudikoff and M. Potter, *Biochemistry* 13, 4033 (1974); S. Rudikoff, Y. Sarow, E. Padian, D. Davics, M. Potter, *Mol. Immunol.* 18, 705 (1981).
16. D. M. Segal *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 71, 4298 (1974).
17. L. Hendershot, D. Bole, J. F. Kearney, *Immunol. Today* 8, 111 (1987).
18. M. S. Briggs and L. M. Gierasch, *Adv. Protein Chem.* 38, 109 (1986).
19. N. R. Morva, K. Nakamura, M. Inouye, *J. Biol. Chem.* 255, 27 (1980).
20. H. Inouye, W. Barnes, J. Beckwith, *J. Bacteriol.* 149, 434 (1982).
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22. C. Eckerskorn, W. Mewes, H. Goretzki, F. Lottspeich, *Eur. J. Biochem.*, in press.
23. H. Metzger, B. Chesebro, N. M. Hadler, J. Lee, N. Otchin, in *Progress in Immunology: Proceedings of the 1st Congress of Immunology* (Academic Press, New York, 1971), pp. 253-267.
24. G. Schumacher, D. Sizmman, H. Haug, P. Buckel, A. Böck, *Nucleic Acids Res.* 14, 5713 (1986).
25. J. R. Hobbs, *Essays Med. Biochem.* 1, 105 (1975).
26. D. Inbar, J. Hochman, D. Givol, *Proc. Natl. Acad. Sci. U.S.A.* 69, 2659 (1972).
27. J. Sen and S. Beychok, *Proteins* 1, 256 (1986).
28. R. Huber and W. S. Bennett, *Nature* 326, 334 (1987); M. Marquart and J. Deisenhofer, *Immunol. Today* 3, 160 (1982).
29. C. Yanisch-Perron *et al.*, *Gene* 33, 103 (1985).
30. A. Skerra and A. Pluckhuhn, in preparation.
31. S. P. Flüg and D. S. Gregerson, *Anal. Biochem.* 155, 83 (1986).

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33. B. Chesebro and H. Metzger, *Biochemistry* 11, 766 (1972).
34. R. K. Scopes, *Protein Purification: Principles and Practice* (Springer, New York, 1982), pp. 241-242.
35. G. Scatchard, *Ann. N.Y. Acad. Sci.* 51, 660 (1949).
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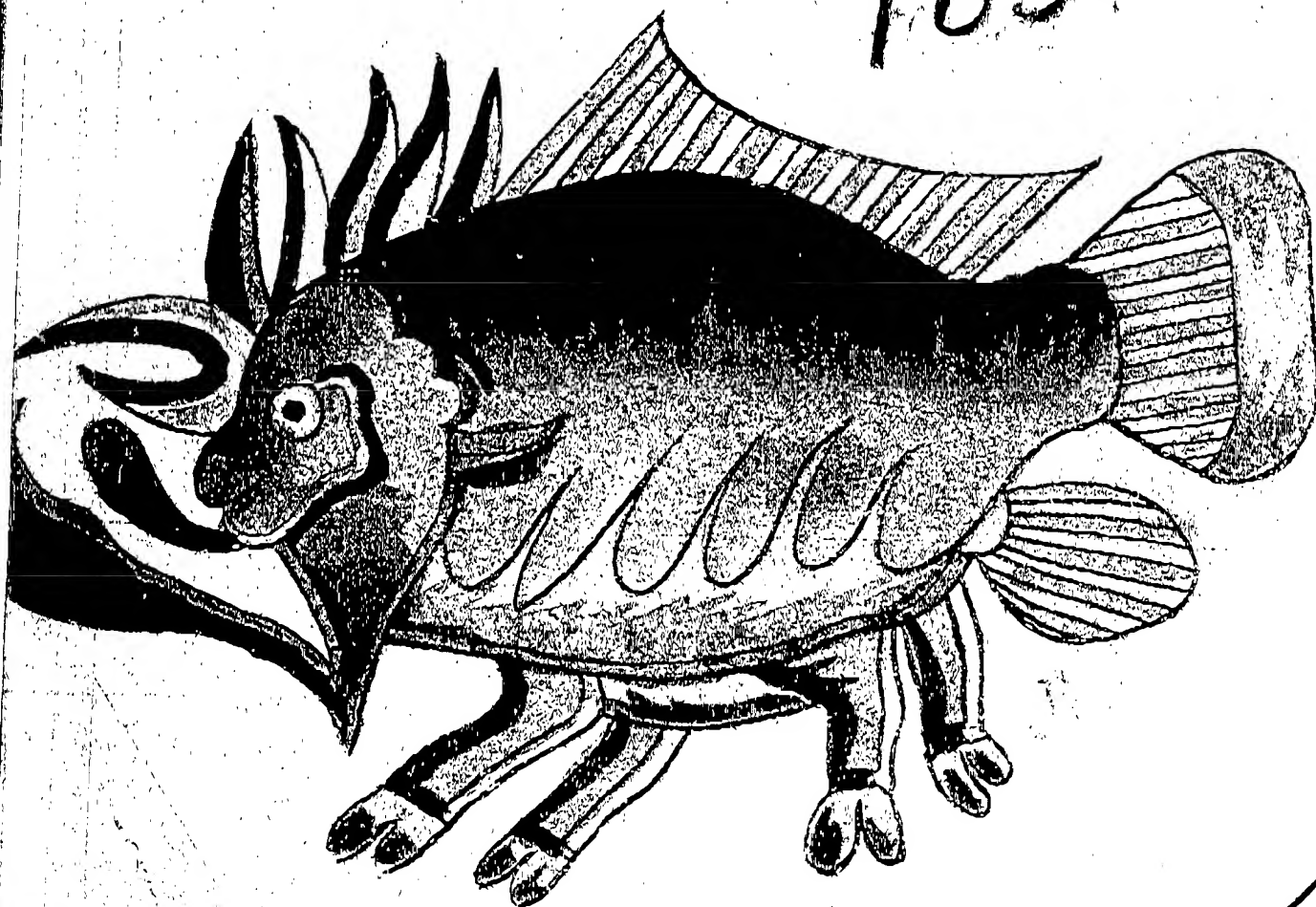
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APPENDIX 3

14. P. T. Jones, P. H. Dear, J. Foote, M. S. Newell, G. Winter, *ibid.* 321, 522 (1986).
15. M. Potter, *Adv. Immunol.* 25, 141 (1977); R. M. Perlmutter *et al.*, *ibid.* 35, 1 (1984); S. Rudikoff and M. Potter, *Biochemistry* 13, 4033 (1974); S. Rudikoff, Y. Satow, E. Padlan, D. Davies, M. Potter, *Mol. Immunol.* 18, 705 (1981).
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also used to insert an Aat II restriction site into the chimeric κ chain gene at the junction of the leader peptide and the mature processed κ chain. Each coding sequence, Fd and κ , was fused to the leader peptide segment of the bacterial *pelB* gene (pectate lyase) from *Erwinia carotovora* (7) generating a gene fusion between the leader peptide segment of the pectate lyase gene and the mature coding sequence of the Fd and κ genes. This bacterial leader sequence was chosen to direct membrane translocation in *E. coli* since pectate lyase can be secreted to high levels under the control of a regulated promoter. To ensure that both Fd and κ were also translated in close physical proximity, we assembled a plasmid that codes both genes in a single dicistronic message (Fig. 1b). This operon was placed under the control of the inducible *araB* promoter from *Salmonella typhimurium* (8) and expressed in *E. coli*.

Examination of culture supernatants or extracts of the periplasmic space (9) of *E. coli* by enzyme-linked immunosorbent assay (ELISA) for chimeric κ with antibody against human κ (Cappel), or Fab production with antibody against human Fd (Calbiochem) and antibody against human κ , revealed that about 90% of the secreted κ chain accumulated in the culture medium. This was a surprising observation that allowed simple purification of this material from induced bacterial cultures. Approximately 2 mg/liter of material reactive as Fab in an ELISA is secreted into culture super-

natants of MC1061 (pTT106). Immunoblot analysis with antibody against human κ revealed that under nonreducing gel conditions, the predominant reactive species had a molecular size of about 48 kD. Under gel conditions where disulfide linkages were reduced, the predominant species had a molecular size of about 23 kD. These observations are consistent with the predicted molecular sizes of the processed chimeric Fab (κ , 23.3 kD; Fd, 24.7 kD), and suggest that the material is properly assembled.

For purification of Fab, bacterial supernatants were concentrated, filtered, and loaded on an SP disk equilibrated with 10 mM phosphate buffer, pH 7.5. Fab was eluted with 0.2M NaCl and purified by S Sepharose column chromatography, where it was eluted as a single peak with a linear 0 to 0.12M NaCl gradient. The immunologically reactive material was more than 90% pure as determined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining (Fig. 2). Purified material has a molecular size of about 48 kD under nonreducing gel conditions and about 24.5 and 23 kD under reducing gel conditions. The 23-kD band is immunologically distinguishable by using antibody against human κ .

Purified bacterial Fab was tested for binding to L6 antigen-containing cells (Table 1). Bacterial Fab bound specifically to the human colon carcinoma cell line C3347; cells from the T cell line T51 served as a negative control. Bacterially produced Fab

also exhibited characteristic binding inhibition of FITC-labeled mouse L6 antibody to the surface of antigen-positive C3347 colon carcinoma cells (Fig. 3). We tested bacterially produced chimeric Fab, proteolytically prepared Fab from L6 mouse antibody, L6 chimeric antibody, and Fab prepared from Sp2/0 cells transfected with the truncated chimeric Fd and the chimeric κ gene. All Fab preparations have essentially identical binding inhibition profiles. The proteolytically produced Fab contains a significant proportion of degraded, low molecular size peptides, whereas chimeric Fab from bacteria or Sp2/0 cells is homogeneous (Fig. 2).

Protein engineering allowed the expression in *E. coli* of a functional chimeric Fab that has binding specificity for a human carcinoma cell marker. The finding that *E. coli* can be engineered to secrete a foreign heterodimeric molecule builds on the earlier report that intrachain disulfide bonds can form correctly in proteins secreted into the periplasmic space of *E. coli* (10). One useful application for bacterially produced Fab molecules will be in tumor imaging in vivo (11, 12) [tumor marker-specific Fabs produced proteolytically from whole antibodies have already been used for this purpose (13-15)]. A great advantage of the engineered bacterial Fab is that the protein heterogeneity that results from nonspecific cleavages and differences in the susceptibility of anti-

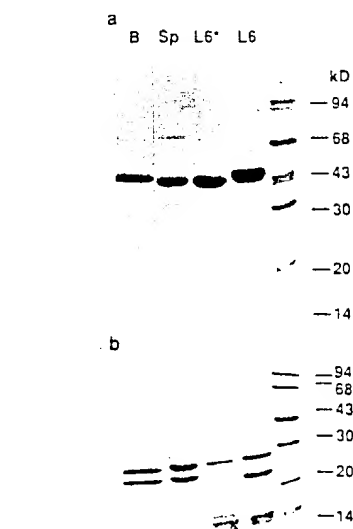
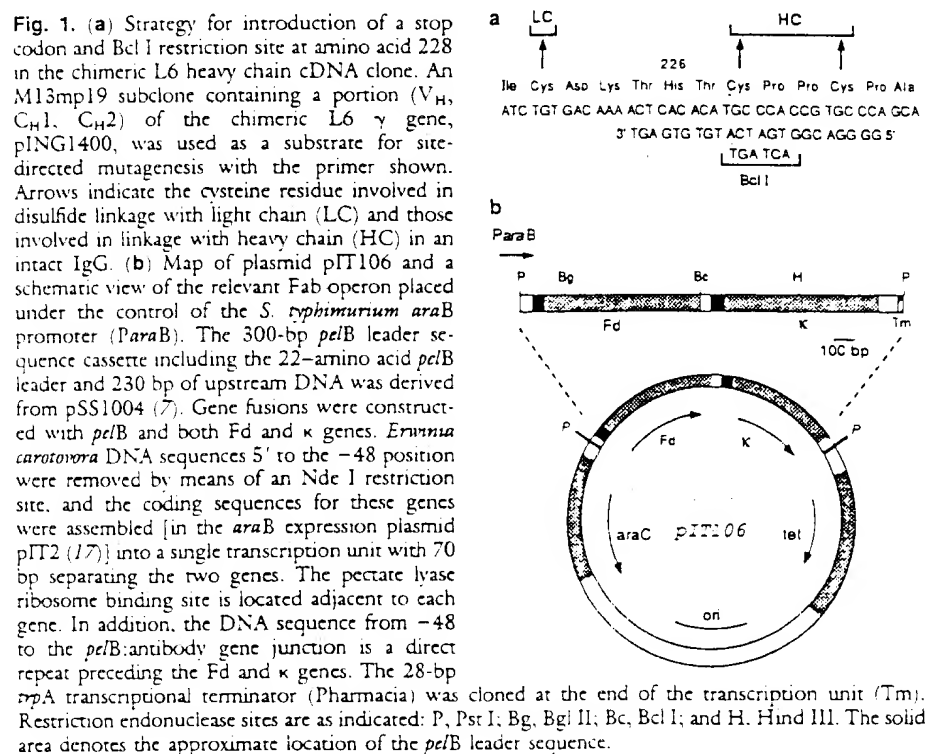


Fig. 2. SDS-PAGE comparison of bacterially (B) and Sp2/0-produced (Sp) Fab to papain-produced mouse L6 and chimeric L6* Fab. Mouse L6 and chimeric L6 antibodies were digested with papain (18), and Fab was purified by S-Sepharose chromatography. Each protein was examined by SDS-PAGE on a 10% gel under nonreducing conditions (a) and on a 12% gel under reducing conditions (b).

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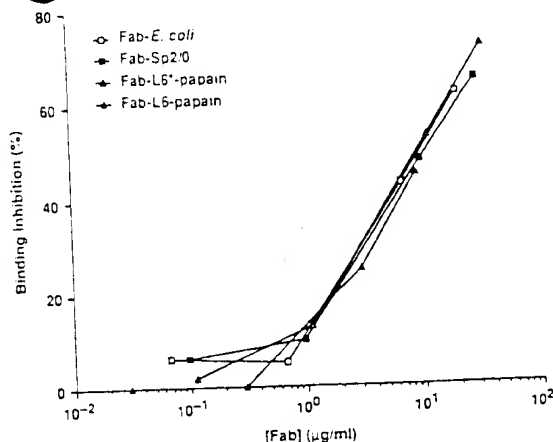
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Fig. 3. Binding inhibition of bacterial Fab, Bacterial Fab, Sp2/0 Fab, and proteolytically produced chimeric L6 Fab (L6*) and mouse L6 Fab were used to inhibit FITC-labeled mouse L6 antibody binding to the surface of antigen-positive C3347 colon carcinoma cells.



bodies to protease cleavage will be obviated; a consistent, homogeneous preparation can be produced. Of additional interest is the relative ease with which the Fab cDNA genes can be modified before expression in bacteria. For example, modifications of the primary structure of either the Fd or κ chain (or both) that are useful for subsequent conjugation of imaging or therapeutic agents or fusion to other peptides (16) can be introduced by site-directed mutagenesis

techniques. We found that *E. coli* can properly assemble a functional two-protein unit with a complex pattern of intra- and inter-chain disulfide linkages and that sufficient quantities of this material may be prepared for eventual use as a human diagnostic and therapeutic reagent.

REFERENCES AND NOTES

1. M. A. Boss, J. H. Kenten, C. R. Wood, J. S. Emtrage, *Nucleic Acids Res.* 12, 3791 (1984).
2. S. Cabilly *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 81,

- 3273 (1984).
3. O. Zemel-Dreazen and A. Zamir, *Gene* 27, 315 (1984).
4. A. Y. Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84, 3439 (1987).
5. I. Hellstrom *et al.*, *Cancer Res.* 46, 3917 (1984).
6. W. Kramer *et al.*, *Nucleic Acids Res.* 12, 9441 (1984).
7. S. P. Lei, H. C. Lin, S. S. Wang, J. Callaway, G. Wilcox, *J. Bacteriol.* 169, 4379 (1987).
8. S. Johnston *et al.*, *Gene* 34, 137 (1985).
9. N. Yanagida, T. Uozumi, T. Beppu, *J. Bacteriol.* 166, 937 (1986).
10. H. M. Hsiung, N. G. Mayne, G. W. Becker, *Biotechnology* 4, 991 (1986).
11. E. Haber, *Annu. Rev. Med.* 37, 249 (1986).
12. J. R. Zalcberg, *Am. J. Clin. Oncol.* 8, 481 (1985).
13. B. Delalove *et al.*, *J. Clin. Invest.* 77, 301 (1986).
14. J. F. Earv *et al.*, in preparation; P. G. Abrams *et al.*, *Second International Conference of Monoclonal Antibody Immunoconjugates for Cancer* (University of California, San Diego Cancer Center, San Diego, 1987), p. 12.
15. R. L. Wahl, D. W. Parker, G. W. Philpott, *J. Nucl. Med.* 24, 316 (1983).
16. E. S. Vitetta *et al.*, *Science* 238, 1098 (1987).
17. L. Masson and D. S. Ray, *Nucleic Acids Res.* 14, 5693 (1986).
18. A. Johnstone and R. Thorpe, *Immunochimistry in Practice* (Blackwell, Oxford, 1982), pp. 48-56.
19. We thank K. E. Hellstrom and co-workers for help performing Fab assays, R. Kodori for the *pelB* gene cassette, R. Rahbar and B. Haselbeck for technical assistance, and R. Wall and G. Wilcox for comments on the manuscript. This work was supported by INGENE.

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Technical Comments

Carcinogenic Risk Estimation

In their widely publicized and popularized article "Ranking possible carcinogenic hazard," Bruce N. Ames *et al.* (17 Apr. 1987, p. 271) conclude that "analysis on the levels of synthetic pollutants in drinking water and of synthetic pesticide residues in foods suggests that this pollution is likely to be a minimal carcinogenic hazard relative to the background of natural carcinogens" and thus that the "high costs of regulation" of such environmental carcinogens are unwarranted. These conclusions reflect both flawed science and public policy.

Although Ames *et al.* challenge the validity of animal carcinogenicity data for quantitative estimation of human risk, they nevertheless use such extrapolations, based on the percentage Human Exposure dose/Rodent Potency dose (HERP), for ranking carcinogenic hazards. Apart from the fact that HERP rankings are based on average population exposures excluding sensitive subgroups, such as pregnant women, the derived potencies of Ames *et al.*, doses inducing tumors in half the tumor-free animals, are misleading. Potencies for "synthetic poi-

lutants," such as trichloroethylene, are derived from bioassays in which lowest doses are large fractions of the maximally tolerated dose (MTD), whereas potencies for more extensively studied "natural carcinogens," such as aflatoxins, are generally derived from titrated doses, orders of magnitude below the MTD. Since dose-response curves are usually flattened near the MTD (1), potencies derived from high-dose testing yield artificially low risk estimates; HERPs for "synthetic" carcinogens are thus substantially underestimated compared with many "natural carcinogens."

Compounding this misconception, Ames *et al.* maintain that carcinogenic dose-response curves rise more steeply than linear curves and that tumor incidences increase more rapidly than proportional to dose. At high doses, dose-response curves are usually less steep than linear curves (1), as also recognized elsewhere by Ames and his colleagues (2). Thus at MTD doses, large further dose increases may induce only small increases in tumor incidence, perhaps reflecting competition between transforma-

tion and cytotoxicity (3); linear extrapolations from high-dose tests thus underestimate low-dose risks.

For Ames *et al.*, the term "carcinogen" heterogeneously includes direct and indirect influences, including promoting and modifying factors and mutagens. Caloric intake is considered "the most striking rodent carcinogen." However, no correlations have been established between food intake and tumor incidence among animals eating ad libitum, despite wide variations in caloric intake and body weight (4), nor have correlations been established between obesity and most human cancers. In the statement by Ames *et al.*, "at the MTD a high percentage of all chemicals might be classified as 'carcinogens'," toxicity and carcinogenicity are confused. However, among some 150 industrial chemicals selected as likely carcinogens and tested neonatally at MTD levels, fewer than 10% were carcinogenic (5). Many highly toxic chemicals are noncarcinogenic, and carcinogen doses in excess of the MTD often inhibit tumor yields. While Ames *et al.* revive the discredited theory that chronic irritation causes cancer, most irritants are noncarcinogenic, and there is no correlation between nonspecific cell injury and carcinogenic potency (6).

Ames *et al.* classify ethanol as carcinogenic, "[one of the two] largest identified causes

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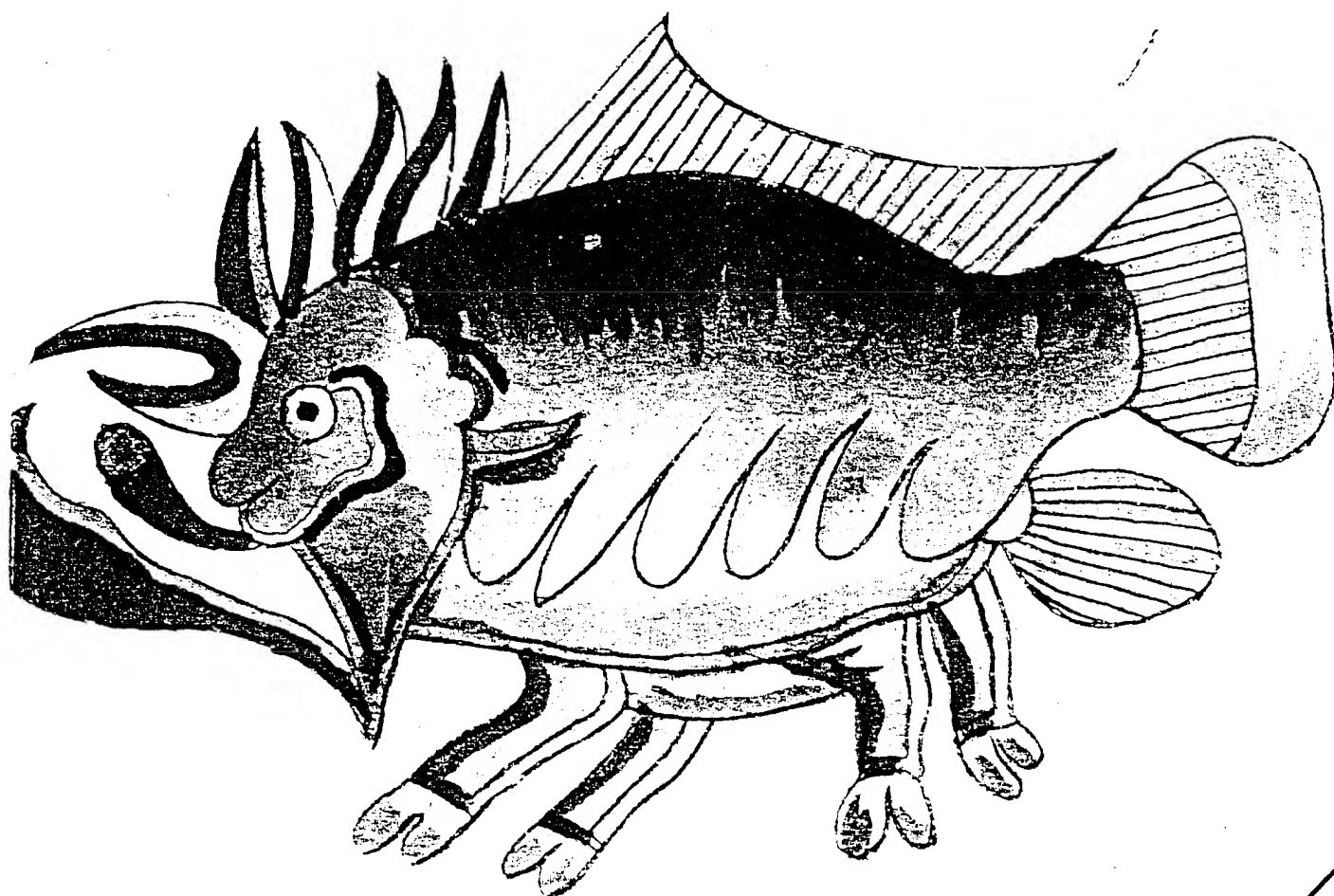
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BOOK ISSUE

APPENDIX 5

Secretion of functional antibody and Fab fragment from yeast cells

(*Saccharomyces cerevisiae*/chimeric antibody/yeast signal sequence/tumor antigen binding/effector functions)

ARNOLD H. HORWITZ^{*†}, C. PAUL CHANG^{*}, MARC BETTER^{*}, KARL ERIK HELLSTROM[†],
AND RANDY R. ROBINSON^{*}

^{*}International Genetic Engineering, Inc., 1545 17th Street, Santa Monica, CA 90404; and [†]Oncogen, 3005 First Avenue, Seattle, WA 98121

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ABSTRACT We have constructed yeast strains that secrete functional mouse-human chimeric antibody and its Fab fragment into the culture medium. For chimeric whole antibody, cDNA copies of the chimeric light-chain and heavy-chain genes of an anti-tumor antibody were inserted into vectors containing the yeast phosphoglycerate kinase promoter, invertase signal sequence, and phosphoglycerate kinase polyadenylation signal. Simultaneous expression of these genes in yeast resulted in secretion of properly folded and assembled chimeric antibody that bound to target cancer cells. Yeast chimeric antibody exhibited antibody-dependent cellular cytotoxicity activity but not complement-dependent cytotoxicity activity. For production of Fab fragments, a truncated heavy-chain (Fd) gene was created by introducing a stop codon near the codon for the amino acid at which papain digestion occurs. Simultaneous expression of the resulting chimeric Fd and light-chain genes in yeast resulted in secretion of properly folded and assembled Fab fragment that bound to target cancer cells.

While a number of single-chain heterologous proteins have been secreted from yeast (1-7), the secretion of foreign multimeric or heterodimeric proteins has not been reported. Of the latter two groups, antibody molecules or the protein fragments that contain their antigen-binding domains, Fab and F(ab')₂ (Fig. 1), are particularly important for a wide variety of applications, including detection and treatment of human disease (8, 9), *in vitro* diagnostic tests (10), and affinity purification methods (11). Chimeric antibodies that consist of mouse variable (V) regions fused to human constant (C) regions may be especially valuable for human therapeutic or *in vivo* diagnostic uses, since they are potentially less immunogenic in humans than are mouse antibodies.

We have described (12) the development of a cDNA cloning strategy for the construction and expression of chimeric antibodies. In this approach, cDNAs coding for the mouse V regions are fused at the immunoglobulin joining (J) region to cDNAs coding for human IgG1 C regions. The cDNA approach for chimeric antibody construction provides an ideal starting point for expression of these genes in microbial systems that either do not undertake mRNA splicing or do so only rarely. In this paper, we describe the secretion from yeast of chimeric antibody and Fab protein. The yeast-secreted molecules (both whole antibody and Fab) bind to target cells as well as their lymphoid cell-derived counterparts. In addition, yeast-derived whole antibody has the same antibody-dependent cellular cytotoxicity (ADCC) activity observed with lymphoid cell-derived whole chimeric antibody but lacks the complement-dependent cytotoxicity (CDC) activity shown by the lymphoid cell-derived antibody.

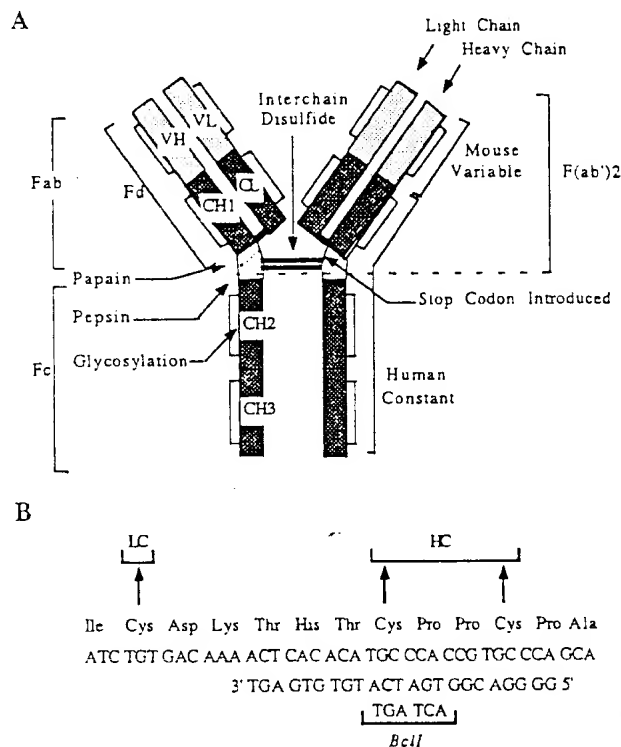


FIG. 1. (A) Structure of mouse-human chimeric IgG1. The locations of papain and pepsin cleavage sites and the structures of Fab and F(ab')₂ resulting from digestion with these enzymes are shown. The locations of N-linked glycosylation and a stop codon introduced by site-directed mutagenesis are also shown. V, variable; C, constant; H, heavy; L, light. (B) Site of *in vitro* mutagenesis and DNA sequence of the mutagenesis primer used to place a stop codon and Bcl I site in the sequence encoding the hinge region of human γ1 heavy chain. The Bcl I site was converted to Xho I by digestion with Bcl I followed by treatment with phage T4 DNA polymerase and ligation with Xho I linkers. The stop codon was unaffected by this treatment. Arrows indicate interchain disulfide bonds with light chain (LC) and heavy chain (HC).

MATERIALS AND METHODS

Strains and Media. *Escherichia coli* strain MC1061 (13) was used as a host for plasmids. *E. coli* strain 71.18 (14) was used as a host for bacteriophage M13. *Saccharomyces cerevisiae* strain BB331C (MATa *ura3 leu2*) was used as a host for yeast transformations performed as described by Ito *et al.* (15). *E. coli* was grown in TYE broth (1.5% Tryptone/1.0% yeast extract/0.5% NaCl) or agar (1.5% Bacto) supplemented, as

needed, with ampicillin (50 $\mu\text{g}/\text{ml}$). Yeast transformants were selected on SD agar (2% glucose/0.67% yeast nitrogen base/2% agar) and grown in SD broth buffered with 50 mM sodium succinate (pH 5.5).

In Vitro Mutagenesis. Site-directed *in vitro* mutagenesis to place restriction sites at yeast or mammalian signal-sequence processing sites and a stop codon in the heavy-chain hinge region was performed as described by Kramer *et al.* (14). Phage plaques containing the desired mutation were identified by plaque-filter hybridization with ^{32}P -labeled primer.

Enzyme-Linked Immunosorbent Assay (ELISA). Light chain was detected by double-antibody sandwich ELISA (16) using goat anti-human κ antiserum as the coating antibody and peroxidase-labeled goat anti-human κ antiserum for quantitation of bound κ protein. Heavy chain was detected similarly with goat anti-human γ antisera with and without peroxidase label. Association of κ light chains and γ heavy chains was detected with goat anti-human κ antiserum as coating antibody and peroxidase-labeled goat anti-human γ antiserum as the second antibody.

Isolation of Chimeric Whole Antibody and Chimeric Fab from Yeast. Whole antibody was purified from the culture supernatant of a 10-liter fermentation as follows. The culture supernatant was concentrated by ultrafiltration (DC10 ultrafiltration system with spiral cartridge, 30-kDa size cutoff; Amicon), filtered through a 0.45- μm filter, and concentrated over a YM30 filter (Amicon) to 250 ml. Antibody protein was purified from the concentrated supernatant by protein A-Sepharose chromatography (11). Analysis of this protein by nonreducing polyacrylamide gel electrophoresis followed by Coomassie blue staining and immunoblotting with anti-human κ antiserum (Sigma) as probe revealed a whole immunoglobulin-size protein band against a background smear. The protein in this band was purified by HPLC on an C_{18} (5- μm particle size) column (Baker), with elution by a linear gradient of 10–125 mM potassium phosphate (pH 6.8).

Fab protein was purified from 1 liter of culture supernatant by concentrating over an Amicon YM30 filter, washing with 130 ml of 10 mM potassium phosphate at pH 7.5 (buffer A), and reconcentrating to 12.5 ml. The supernatant was diluted

to 54 ml with buffer A and loaded onto a 1.5-ml S-Sepharose column, washed with 20 ml of buffer A, and eluted with a 40-ml linear gradient of 0–200 mM NaCl in buffer A.

Fab protein prepared by papain digestion (11) of 3 mg of whole L6 mouse antibody or chimeric antibody was purified on a 25-ml S-Sepharose column by elution with an 80-ml linear gradient of 0–120 mM NaCl prepared in 10 mM sodium phosphate (pH 7.5). The Fab protein was eluted at 60 mM NaCl and was free of Fc protein.

Functional Tests of Chimeric Antibody and Fab from Yeast. The following tests were used to assess function: (i) direct binding of whole antibody or Fab to target cells that are positive or negative for the L6 antigen; (ii) competition inhibition of binding of L6 mouse antibody to antigen-positive cells; (iii) ADCC and CDC assays with whole antibody. The binding assays were performed with a Coulter model EPIC-C cell sorter (17). ADCC and CDC assays were performed with ^{51}Cr -labeled target cells (18, 19) that were exposed to the antibodies and peripheral blood leukocytes or human serum over a period of 4 hr.

RESULTS

Construction of Yeast Expression Plasmids Containing Antibody Genes. To facilitate light- and heavy-chain secretion from yeast, the gene sequences encoding the mature forms of the light and heavy chains of a chimeric anti-tumor antibody (L6, ref. 12) were fused to the yeast invertase signal sequence (20) and placed under the control of the phosphoglycerate kinase (PGK) gene promoter (21). These fusions were then cloned into yeast expression vectors containing the PGK polyadenylation signal (21) to generate pING1441 (light chain, *leu2*) and pING1442 (heavy chain, *ura3*) (Fig. 2A).

The Fd portion of heavy chain consists of the V region and the $\text{C}_{\text{H}2}$ domain (Fig. 1) and is generated by digestion of whole antibody with papain (11). To enable yeast to synthesize Fd protein, a stop codon was introduced by site-directed mutagenesis of the chimeric heavy-chain gene so that translation would terminate in the hinge region, near the papain recognition site (Fig. 1B). A *Bcl* I site was introduced along

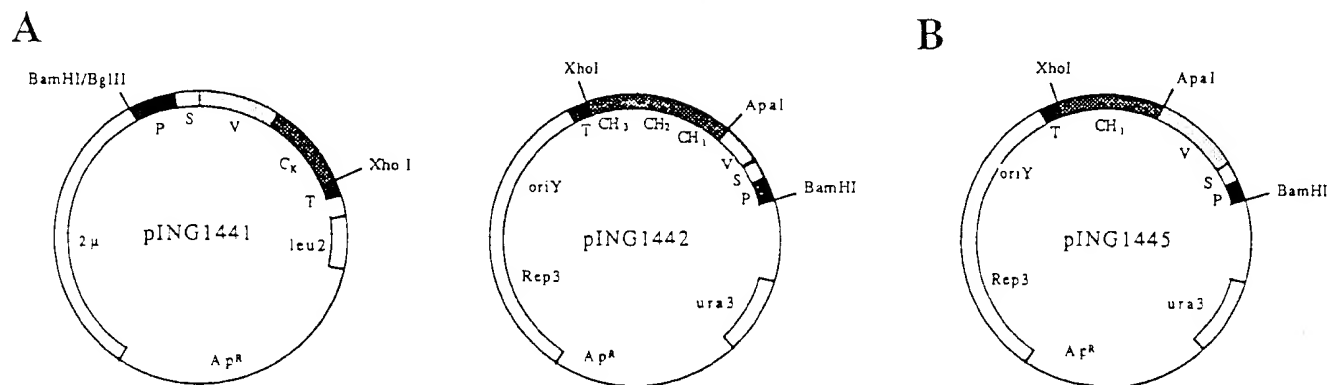


FIG. 2. Structure of yeast immunoglobulin expression plasmids. The fusions of the gene sequences encoding the mature forms of light and heavy chain to the yeast invertase signal sequence and PGK promoter were accomplished by first introducing by *in vitro* mutagenesis a unique restriction site at the signal-sequence processing sites for both the invertase signal sequence (*Pst* I) and the light (*Aat* II) and heavy (*Sst* I) chain genes. These sites were positioned such that a blunt-ended ligation of restriction enzyme-digested, T4 DNA polymerase-treated DNAs resulted in in-phase translational fusions of the 5' end of the mature immunoglobulin chains with the 3' end of the yeast invertase signal sequence. (A) The light-chain expression plasmid, pING1441, was constructed by cloning a *Bgl* II-*Xho* I fragment containing the light-chain gene into a yeast expression vector, pING804CVS (provided by J.-H. Lee, International Genetic Engineering). pING1441 contains the complete 2- μm plasmid (2 μ); the chimeric light-chain gene (V and C_{L} regions) fused to the PGK promoter (P), invertase signal sequence (S), and PGK transcription termination and polyadenylation signals (T); and the *leu2* gene as the yeast selective marker. The heavy-chain expression plasmid, pING1442, constructed by cloning a *Bam* HI-*Xho* I fragment containing the heavy-chain gene into a yeast expression vector, pING1150. pING1442 contains the yeast origin of replication (*ori*Y) and a cis-acting stabilization sequence (Rep3) from yeast 2- μm plasmid; the chimeric heavy-chain gene (V-region and C-region domains $\text{C}_{\text{H}1}$, $\text{C}_{\text{H}2}$, and $\text{C}_{\text{H}3}$) fused to the PGK promoter, invertase signal sequence, and PGK transcription termination and polyadenylation signals; and the *ura3* gene as the yeast selective marker. (B) The Fd-chain expression plasmid, pING1445, is identical to the heavy-chain expression plasmid, pING1442, with the exception that the *Apa* I-*Xho* I restriction fragment in pING1442 encoding $\text{C}_{\text{H}1}$, $\text{C}_{\text{H}2}$, and $\text{C}_{\text{H}3}$ has been replaced with a fragment encoding only $\text{C}_{\text{H}1}$.

with the stop codon. Following conversion of the *Bcl* I site to *Xho* I, the *Apa* I-*Xho* I C_H1 -region fragment of pING1442 (Fig. 2A) was replaced with an *Apa* I-*Xho* I C_H1 fragment containing the stop codon in the hinge region to generate pING1445 (Fig. 2B).

Secretion of Whole Chimeric Antibody from Yeast. The plasmids pING1441 and pING1442 were cotransformed into *S. cerevisiae* strain BB331C by selection for Ura⁺ Leu⁺ colonies. Ten transformants were grown for 3 days in 5 ml of SD broth and the culture supernatants were analyzed by ELISA for the levels of light chain, heavy chain, and associated light and heavy chains. The culture supernatants of two transformants (nos. 1 and 5) contained light chain at ≈ 100 ng/ml and heavy chain at 50–80 ng/ml, and 50–70% of the heavy chain was associated with light chain. These proteins were concentrated on a Centricon 30 filter (Amicon), electrophoresed in a NaDodSO₄/7% polyacrylamide gel under nonreducing conditions, and transferred to nitrocellulose. κ -crossreactive protein was detected with goat anti-human κ antiserum followed by peroxidase-labeled rabbit anti-goat antiserum. A faint, but distinct, band that comigrated with purified chimeric L6 antibody produced by Sp2/0 cells was observed in the lanes containing both supernatants (data not shown). These results suggested that the yeast transformants were synthesizing and secreting a fully assembled chimeric antibody that was very similar to lymphoid cell-derived antibody.

To prepare sufficient quantities of yeast-derived antibody for detailed characterization of structure and function, transformant no. 5 was grown in a 10-liter fermentor for 58 hr. Whole antibody was purified from this culture medium as described in *Materials and Methods*. ELISA analysis of column fractions from HPLC (AB_x 5- μ m) revealed a heavy (γ) and light (κ) chain-crossreactive peak corresponding to a distinct A₂₈₀ peak. Analysis of these fractions by nonreducing NaDodSO₄/polyacrylamide gel electrophoresis revealed a protein that comigrated with L6 chimeric antibody purified from Sp2/0 cells (Fig. 3). Under reducing conditions, the protein in these fractions was resolved into a light-chain band, which comigrated with the light chain of L6 chimeric antibody from Sp2/0 cells, and two heavy chain bands, which migrated near the heavy chain from Sp2/0 cells (Fig. 3). This purified preparation of yeast-produced chimeric antibody was used in further binding and function assays.

Secretion of Chimeric Fab from Yeast. The plasmids pING1441 (Fig. 2A) and pING1445 (Fig. 2B) were cotransformed into *S. cerevisiae* BB331C and the transformants were grown in broth under selective conditions as described above. The culture supernatants were assayed by ELISA and were found to contain light chain at 100–200 ng/ml. To determine whether the cells secreted a Fab-size protein, the culture supernatants were concentrated with Centricon 30 filters, electrophoresed in a nonreducing NaDodSO₄/10% polyacrylamide gel, and electrophoretically transferred to nitrocellulose paper. Light chain-crossreactive protein was detected with goat anti-human κ antiserum followed by peroxidase-labeled rabbit anti-goat antiserum. One of the five transformants secreted a distinct κ -crossreactive band, which migrated at the expected position for Fab protein (data not shown).

The yeast strain that secreted the Fab-size protein was grown in 1 liter of SD broth for 4 days at 30°C and Fab protein was purified from the culture supernatant. Nonreducing NaDodSO₄/polyacrylamide gel analysis of pooled S-Sepharose column fractions containing anti-human κ -crossreactive protein revealed a 46-kDa protein, comigrating with chimeric Fab prepared by papiin digestion of Sp2/0 cell-derived chimeric whole antibody (Fig. 4). Electrophoresis under reducing conditions resolved this protein into two bands that migrated at 23 and 25 kDa (Fig. 4). The 23-kDa

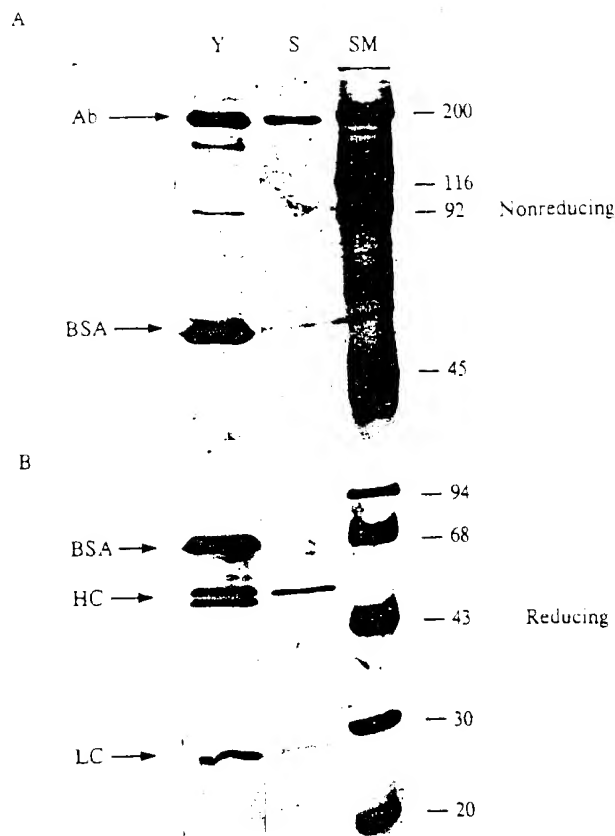


FIG. 3. Silver-stained NaDodSO₄/polyacrylamide gels showing purified chimeric antibody secreted by yeast. (A) Nonreducing 7% gel. (B) Reducing 10% gel. Intensely stained band at 68 kDa on both gels is bovine serum albumin (BSA), which was present as a carrier protein. Size marker (lanes SM) molecular masses (in kDa at right) and relevant yeast-derived (lanes Y) and Sp2/0-derived (lanes S) antibody bands [Ab on nonreducing gel; HC (heavy chain) and LC (light chain) on reducing gel] are identified.

band was identified as light chain by its reactivity on an immunoblot with anti-human κ antiserum. These results were consistent with the predicted molecular masses, based on nucleotide sequence, for fully processed L6 chimeric light chain (23.3 kDa) and Fd chain (24.8 kDa).

Binding Characteristics of Chimeric Whole Antibody and Fab Protein Secreted by Yeast. The purification from yeast culture supernatants of protein of the expected size of whole antibody and Fab suggested that the yeast transformants were secreting correctly folded, functional molecules. This hypothesis was confirmed by performing direct and competition binding assays with a human colon carcinoma cell line (line C-3347) that expresses 5×10^5 molecules of the L6 tumor antigen per cell (17). In the direct binding assay, both whole antibody and Fab from yeast bound to the target cancer cells but not to a control cell line that lacked the L6 antigen (data not shown). In the competition assay using mouse L6 antibody, 50% binding inhibition was observed at the same concentration (2 μ g/ml) for both yeast-derived and Sp2/0 cell-derived whole chimeric antibody (Fig. 5). In the same assay, the yeast-derived L6 chimeric Fab behaved identically to both Sp2/0 cell-derived chimeric and mouse Fab proteins prepared by papiin digestion (Fig. 5). Fifty percent inhibition of mouse L6 antibody was achieved by the yeast-derived Fab at 7 μ g/ml.

Chimeric Whole Antibody from Yeast Mediates ADCC but Not CDC. Two additional tests for function were performed with chimeric whole antibody from yeast: (i) mediation of ADCC in the presence of human peripheral blood leukocytes

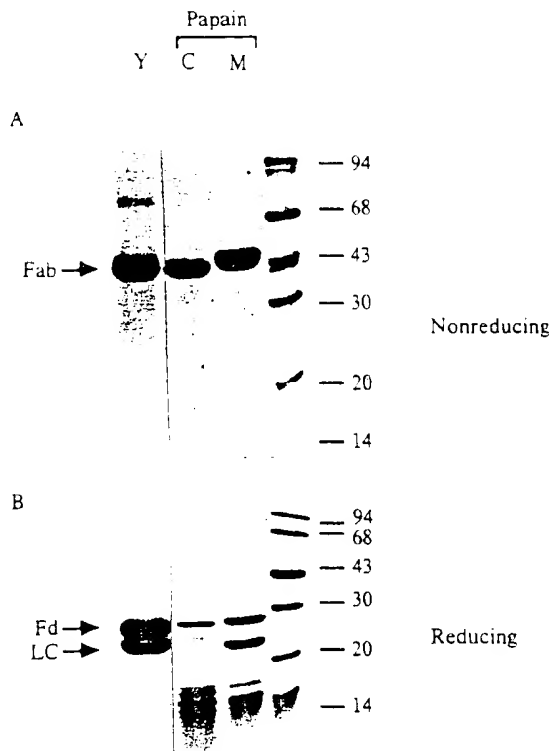


FIG. 4. Coomassie blue-stained gel comparing purified chimeric Fab protein secreted by yeast (lanes Y) with chimeric (lanes C) and mouse (lanes M) Fab fragments produced by papain digestion of intact antibody. (A) Nonreducing 10% gel. (B) Reducing 12% gel. Size marker molecular masses (kDa) and relevant bands are shown. LC, light chain.

and (ii) mediation of tumor-cell lysis in the presence of human complement. ADCC activity of yeast-derived chimeric L6 antibody was slightly higher than that of Sp2/0 cell-derived chimeric antibody, and the ADCC activities of both chimeric antibodies were higher than that of the mouse L6 antibody (Table 1). Yeast-derived L6 chimeric antibody failed to mediate CDC, even at the highest antibody concentration, whereas Sp2/0 cell-de-

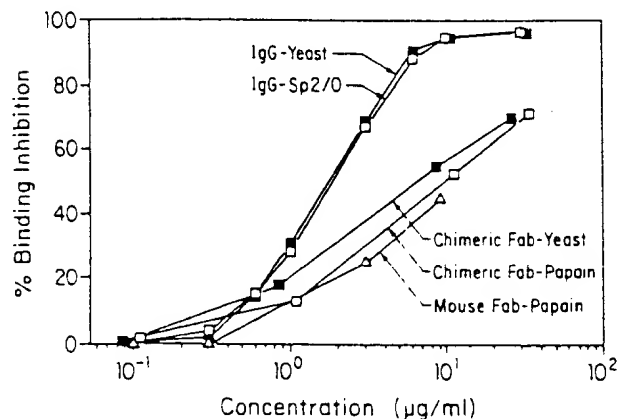


FIG. 5. Comparison in antibody-competition binding assays between whole chimeric L6 antibody (IgG) derived from yeast and Sp2/0 cells, chimeric L6 Fab derived from yeast or prepared by papain digestion of whole chimeric antibody isolated from Sp2/0 cells and mouse Fab prepared by papain digestion of L6 antibody. C-3347 colon carcinoma cells were incubated with various concentrations of unlabeled blocking antibodies before addition of fluorescein isothiocyanate-conjugated mouse L6 antibody (3 µg/ml). Inhibition was measured by flow cytometry.

Table 1. ADCC analysis

Antibody	Conc., µg/ml	% cytotoxicity
Standard mouse L6	5.0	42
	1.0	48
	0.1	71
	0.01	54
Sp2/0 chimeric L6	0.001	37
	1.0	114
	0.1	108
	0.01	76
Yeast chimeric L6	0.001	60
	0	23
None		

The colon carcinoma target cells (line C-3347) were labeled with ⁵¹Cr and exposed for 4 hr to a combination of monoclonal antibody and human peripheral blood leukocytes (100 per target cell), and the release of ⁵¹Cr was measured subsequently. The release of ⁵¹Cr (after corrections of values for spontaneous release from untreated cells) is a measure of cytotoxicity.

Yeast-derived chimeric L6 antibody and mouse L6 antibody exhibited the expected cytotoxic activity (Table 2).

DISCUSSION

We have engineered the yeast *S. cerevisiae* to secrete functional mouse-human chimeric antibody and Fab protein into the culture medium. This was accomplished by simultaneous expression of the mature light-chain gene and the heavy-chain gene or a truncated heavy-chain (Fd) gene fused to the yeast invertase signal sequence and the PGK promoter and polyadenylation signal. Several lines of evidence support the thesis that these proteins are correctly folded. (i) Proteins of the expected size for whole antibody and Fab were purified from the culture supernatants of yeast cells expressing the chimeric light- and heavy- or Fd-chain genes (Figs. 3 and 4). (ii) The whole antibody and Fab from yeast behaved indistinguishably from their lymphoid cell-derived counterparts in both direct and competition binding assays (Fig. 5). (iii) The chimeric whole antibody from yeast exhibited the same ADCC activity as the chimeric antibody from Sp2/0 cells (Table 1).

There have been a number of reports of secretion from yeast of heterologous proteins fused to yeast signal sequences (2, 4, 5, 7). All of the proteins in these examples were composed of a single polypeptide chain. Although there is one example of functional mouse antibody (IgM) production in yeast, the antibody in this case was found only intracellularly in vacuoles; only unassociated light and heavy chains were detected in the culture supernatant (22).

Table 2. CDC analysis

Antibody	Conc., µg/ml	Complement*	% cytotoxicity†
Standard mouse L6	5	+	122
	1	+	53
	5	-	1
Sp2/0 chimeric L6	5	+	73
	1	+	22
	0.1	+	5
	5	-	2
Yeast chimeric L6	5	+	3
	1	+	2
	0.1	+	4

*Human serum from a healthy subject was used as the source of complement.

†⁵¹Cr-labeled C-3347 cells were exposed to human complement and antibody. CDC was measured by a 4-hr ⁵¹Cr-release assay.

The chimeric whole antibody from yeast mediated ADCC in an identical fashion to Sp2/0 cell-derived chimeric antibody (Table 1). These results suggest that the yeast-derived antibody is equivalent to the lymphoid cell-derived antibody in interacting with Fc receptors on killer cells and activating them to mediate ADCC. Interestingly, the chimeric mouse-human antibody from either lymphoid cells or yeast was more efficient at ADCC than was the mouse antibody. Thus, the Fc-receptor interaction involved in ADCC appears to be primarily determined by the amino acid sequence of the Fc portion of the antibody and is probably not affected by the altered glycosylation patterns expected for yeast-derived antibody. This conclusion is consistent with the observation that binding of Fc receptor type I by IgG, which may play a central role in ADCC (23), occurs in the region linking the C_{H2} domain to the hinge (24). This region of the protein is an exposed, flexible strand (24) and is well removed from the site of N-linked glycosylation (25).

An intriguing result is that the yeast-derived antibody lacks the ability to activate complement to lyse target cells (Table 2). This may reflect differences in the glycosylation patterns of the yeast-derived and Sp2/0 cell-derived chimeric antibodies. Several observations support this hypothesis. Binding of complement component C1q, which initiates CDC, occurs within the C_{H2} domain of human IgG (26), which is also the region of N-linked glycosylation. Second, elimination of the N-linked glycosylation site in mouse IgG2b by *in vitro* mutagenesis of asparagine-297 to alanine results in reduced affinity of the antibody for human C1q and complete loss of CDC (27). Third, while yeast and mammalian cells recognize the same peptide signal [Asn-Xaa-(Ser/Thr)] for glycosylation and utilize a similar pathway for core oligosaccharide synthesis in the endoplasmic reticulum (28), the type and extent of outer chain glycosylation appear to be quite different in these cell types (29). Indeed, comparison of yeast- and Sp2/0 cell-derived heavy chains by reducing NaDodSO₄/polyacrylamide gel electrophoresis revealed differences in protein mobility (Fig. 4), which may be caused by differences in glycosylation. Such glycosylation differences may be sufficient to cause a loss of CDC activity. Further studies will be required to establish the exact cause of these size differences and their possible relationship to CDC activity.

Fab proteins may be especially useful for certain diagnostic and therapeutic applications, including *in vivo* tumor imaging (8) and drug or toxin delivery to tumors. Mouse-human chimeric Fab proteins are particularly attractive for these uses because they may be less immunogenic than mouse Fab proteins in humans. Current methods for Fab production involve papain digestion of purified whole antibody (11). This approach can be problematic, since not all antibodies are equivalent in their susceptibility to papain cleavage (11) and additional purification steps are required beyond that for the whole antibody. In addition, papain digestion can result in partial degradation of the Fab (Fig. 4). By contrast, direct production of Fab by yeast or, as described elsewhere, by *E. coli* or Sp2/0 cells (30) can yield a highly purified, homogeneous Fab preparation that has the same binding activity as the Fab prepared by papain digestion (Fig. 5). Further, the introduction by site-directed mutagenesis of a restriction site in conjunction with the stop codon in the Fd sequence permits manipulations at the 3' end of the gene that can yield Fab proteins with altered properties, such as enhanced affinity in labeling reactions, or allow direct production of Fab molecules linked to various proteins.

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- Hitzeman, R. A., Leung, D. W., Perry, L. J., Kohr, W. J., Levine, H. L., & Goeddel, D. V. (1983) *Science* 219, 620-625.
- Brake, A. J., Merryweather, J. P., Coit, D. G., Heberlein, U. A., Masiarz, F. R., Mullenbach, G. T., Urden, M. S., Valenzuela, P., & Barr, P. J. (1984) *Proc. Natl. Acad. Sci. USA* 81, 4642-4646.
- Innis, M. A., Holland, M. J., McCabe, P. C., Cole, G. E., Wittman, V. P., Tal, R., Watt, W. K., Gelfand, D. H., Holland, J. P., & Meade, J. H. (1985) *Science* 228, 21-26.
- Smith, R. A., Duncan, M. J., & Moir, D. T. (1985) *Science* 229, 1219-1224.
- Chang, C. N., Matteucci, M. L., Perry, J., Wulf, J. J., Chen, C. Y., & Hitzeman, R. A. (1986) *Mol. Cell. Biol.* 6, 1812-1819.
- Jigami, Y., Muraki, M., Harada, N., & Tanada, H. (1986) *Gene* 43, 273-279.
- Moir, D. T. & Dumais, D. R. (1987) *Gene* 56, 209-217.
- Delaloy, B., Bischof-Delaloy, A., Buchegger, F., Von Fliedner, V., Grob, J.-P., Volant, J.-C., Pettavel, J., & Mach, J.-P. (1986) *J. Clin. Invest.* 77, 301-311.
- Levy, R. & Millre, R. A. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 42, 2650-2656.
- Tam, M. R., Goldstein, L. C., & Yelton, D. E. (1985) in *Manual of Clinical Microbiology*, eds. Lennette, E. H., Balows, A., Hausler, W. J., Jr., & Shadomy, H. J. (Am. Soc. Microbiol. Washington, DC), 4th Ed., pp. 905-909.
- Johnstone, A. & Thorpe, R. (1982) *Immuno Chemistry in Practice* (Blackwell, Oxford), pp. 52-53.
- Liu, A. Y., Robinson, R. R., Hellstrom, K. E., Murray, E. D., Jr., Chang, C. P., & Hellstrom, I. (1987) *Proc. Natl. Acad. Sci. USA* 84, 3439-3443.
- Casadaban, M. J. & Cohen, S. N. (1980) *J. Mol. Biol.* 138, 179-207.
- Kramer, W., Drutsa, V., Jansen, H.-W., Kramer, B., Pflugfelder, M., & Fritz, H.-J. (1984) *Nucleic Acids Res.* 12, 9441-9456.
- Ito, H., Fukuda, Y., Murata, K., & Kimura, A. (1983) *J. Bacteriol.* 153, 163-168.
- Voller, A., Bidwell, D., & Bartlett, A. (1980) in *Manual of Clinical Immunology*, eds. Rose, N. R., & Friedman, H. (Am. Soc. Microbiol., Washington, DC), 2nd Ed., pp. 359-371.
- Hellstrom, I., Horn, D., Linsley, P., Brown, J. P., Brankovan, V., & Hellstrom, K. E. (1986) *Cancer Res.* 46, 3917-3923.
- Hellstrom, I., Brankovan, V., & Hellstrom, K. E. (1985) *Proc. Natl. Acad. Sci. USA* 82, 1499-1502.
- Hellstrom, I., Beaumier, P. L., & Hellstrom, K. E. (1986) *Proc. Natl. Acad. Sci. USA* 83, 7059-7063.
- Taussig, R. & Carlson, M. (1983) *Nucleic Acids Res.* 11, 1943-1954.
- Hitzeman, R. A., Hagie, F. E., Hayflick, J. S., Chen, C. Y., Seeburg, P. H., & Derynck, R. (1982) *Nucleic Acids Res.* 10, 7791-7808.
- Wood, C. R., Boss, M. A., Kenten, J. H., Calvert, J. E., Roberts, N. A., & Emtage, J. S. (1985) *Nature (London)* 314, 446-449.
- Unkeless, J. C., Scigliano, E., & Freedman, V. H. (1988) *Annu. Rev. Immunol.* 6, 251-281.
- Duncan, A. R., Woof, J. M., Partridge, L. J., Burton, D. R., & Winter, G. (1988) *Nature (London)* 332, 563-564.
- Jeske, D. J., & Capra, J. D. (1984) in *Fundamental Immunology*, ed. Paul, W. E. (Raven, New York), pp. 131-165.
- Yasmeen, D., Ellerson, J. R., Dorrington, K. J., & Painter, R. (1976) *J. Immunol.* 116, 1664-1667.
- Duncan, A. R. & Winter, G. (1988) *Nature (London)* 332, 738-740.
- Schekman, R. (1985) *Annu. Rev. Cell Biol.* 1, 115-143.
- Kukuruzinska, M. A., Bergh, M. L. E., & Jackson, B. J. (1987) *Annu. Rev. Biochem.* 56, 915-944.
- Better, M., Chang, C. P., Robinson, R. R., & Horwitz, A. H. (1988) *Science* 240, 1041-1043.

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